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13. ABSTRACT (Maximum 200 Words)

We have previous demonstrated that Gadd45, a p53-regulated stress protein that plays an important role in cell cycle G2-M checkpoint, is transcriptionally up-regulated by BRCA1. In this report, we have shown that BRCA1-induced cell cycle G2-M arrest is disrupted in Gadd45 deficient cells, indicating that Gadd45 mediates BRCA1's role in the control of cell cycle G2-M arrest. In agreement with this finding, disruption of endogenous of Gadd45 in cells is found to result in impaired capability of BRCA1 in growth suppression. These results further support Gadd45 as a BRCA1 downstream effector gene. In addition, we have demonstrated that BRCA1 activation of the Gadd45 gene is mediated through OCT-1 and CAAT motifs that localize at the Gadd45 promoter. BRCA1 protein can directly bind to the Gadd45 promoter and this binding is mediated via the associations of BRCA1 with Oct-1 and NF-YA transcription factors. Importantly, ATR kinase is found to be involved in the signaling pathway that mediates BRCA1 activation of the Gadd45 promoter. The current study demonstrates a novel pathway (BRCA1-GADD45) involved in cellular response to DNA damage, particularly in the control of cell cycle checkpoint.

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1. INTRODUCTION

Breast cancer is the most frequent malignancy in women. More than half of the hereditary breast cancer can be attributed to mutations in the breast cancer susceptibility gene BRCA1 (1-3). A numbers of investigation demonstrate that that BRCA1 plays an important role in maintenance of genomic fidelity probably through its role in the control of cell cycle checkpoints, DNA repair and apoptosis (4-10). Deregulation of genomic fidelity is closely associated with malignant transformation and tumorigenesis. However, the molecular mechanism by which BRCA1 plays a role in maintaining genomic integrity remains to be defined. Particularly, the signaling pathway that mediates BRCA1-induced cell cycle checkpoint is unclear. The current project is to define the molecular pathway, which mediates BRCA1's role in the control of cell cycle G2-M checkpoint. The proposed studies will provide the understanding of how BRCA1 participates in maintenance of genomic stability and prevents the onset of breast cancer, as well as provide the insight to development of novel new anticancer drugs. Our previous and current work indicates that BRCA1 transcriptionally activates Gadd45, a p53-regulated and DNA damage-inducible gene that may play an important role in cell cycle G2-M checkpoints, apoptosis and DNA repair in response to DNA damage (11-17). We speculated that the role of BRCA1 in cell cycle G2-M checkpoint is mediated through Gadd45. Therefore, two major tasks have been proposed in this study. (1). To define the role Gadd45 in BRCA1-induced cell cycle G2-M arrest. (2). To determine the biochemical and molecular mechanism by which BRCA1 regulates Gadd45.

2. BODY

This is the end of the third year of this grant. Overall, this DOD-funded project has been going well during the past three years The findings from this project have greatly demonstrated a novel pathway (BRCA1-Gadd45) in cellular response to DNA damage, indicating that the role of BRCA1 in the maintenance of genomic stability may be mediated through Gadd45, a DNA damage-inducible gene that plays a role in the cell cycle checkpoint. Furthermore, multiple papers have been generated for this particular project over the last three years.

However, to entirely accomplish the all tasks proposed in this project, we have applied for one year no-cost extension (from August 1, 2003-July 31, 2004). This is due to the fact that some cell lines (Gadd45 deficient cells) we intended to utilize were relied on the resources outside of our lab and we just recently obtained those lines. These cells will be used to examine the hypothesis that Gadd45 mediates BRCA1's growth suppressive property. In addition, we recently found two BRCA1-assocated proteins, which might synergistically work together with Gadd45. We think that the completion of this work would extend our understanding of how BRCA1-Gadd45 pathway acts in maintenance of genomic integrity.

Currently, the application of one-year no-cost extension has been approved by DOD grant agency. According the instruction from DOD grant administration, we are required to submit an annual report at this time.

During the third year of effort on this grant, significant progress has been achieved. These will be summarized as the following individually.

In Task 1:

1. We have previously developed two GADD45-inducible cell lines via tet-off system in Hela (p53 negative status) and HCT116 (with wt p53) cells. In these cell lines, expression of Gadd45 protein is precisely controlled through the presence of tetracycline. We have found that induction of Gadd45 protein in HCT116 cell line (wt p53) results in cells arrest in G2-M phase, but induction of Gadd45 protein in Hela cells (deleted p53) failed to generate G2-M accumulation, suggesting that the Gadd45-activated cell cycle G2-M arrest is dependent of normal cellular p53 function. These results demonstrate that Gadd45, as a BRCA1-targeted effector, is capable of mediating BRCA1-induced cell cycle arrest.

   In addition, we have found UV-induced G2-M checkpoint is impaired in BRCA1-deficient cells. To demonstrate this, we have established isogenic cell lines, where BRCA1 protein is suppressed by expression of antisense BRCA mRNA. In those cell lines, BRCA1 protein expression was shown to be greatly inhibited. Following UV radiation treatment, the accumulation of cells in G2-M phase was substantially reduced, indicating an impaired cell cycle G2-M arrest by UV in BRCA1-deficient cells. We also examined expression of Gadd45 mRNA and found induction of Gadd45 after UV treatment was greatly reduced in BRCA1-deficient cells.

   We Further analyzed Cdc2/cyclin B1 kinase activity in BRCA1-deficient cells exposed to UV and found less inhibition of Cdc2/cyclin B1 activity in such cells compared with their parental cells, suggesting disruption of BRCA1 in cells results in impaired induction of Gadd45 and in turn affects formation of cell cycle G2-M checkpoint.

2. We further demonstrate that BRCA1-induced G2-M checkpoint is impaired in Gadd45 deficient cells. We utilized two different Gadd45-deficient cells. First we introduced BRCA1expression vectors into Gadd45 expressing antisense cells (human cancer cells), where Gadd45 expression is significantly blocked by expression of Gadd45 antisense mRNA, and analyzed cell cycle distribution. We found that BRCA1-activated cell cycle arrest is disrupted in Gadd45 expressing antisense cells. Second, we repeated similar experiments in Gadd45 knockout cells (ras-immortalized embryonic mouse fibroblast derived from gadd45 knockout mice) and found BRCA1-induced G2-M arrest was deficient. In support those findings, we examined Cdc2 kinase activity following BRCA1 introduction and found that disruption of endogenous Gadd45 will greatly remove the BRCA1-mediated Cdc2 kinase inhibition.

3. We have demonstrated that Gadd45 is able to mediate BRCA1-induced growth suppression. We introduced BRCA1 expression vectors into Gadd45 deficient cells (both human line and MEFs) and observed that BRCA1-generated cell growth suppression is partially abrogated in Gadd45-deficient cells.

Taken all together, we have demonstrated that Gadd45 is an important component that mediating BRCA1-activated cell cycle checkpoint and BRCA1-induced cell growth suppression.

In Task 2:

1. In our previous report, we have demonstrated that expression of BRCA1 is able to upregulate Gadd45 mRNA when BRCA1 expression vector is introduced into human breast carcinoma MCF-7 cells. In agreement with this finding, we have found that BRCA1 activates the Gadd45 promoter and BRCA1 activation of the Gadd45 promoter requires normal transcriptional activity of BRCA1 since the tumor-derived mutants of BRCA1, which lack normal transcriptional property, are unable to induce the Gadd45 promoter. Interestingly, BRCA1 activation of the Gadd45 promoter is a BRCA1-mediated specific effect and in a p53-independent manner.

   With regard to the biochemical mechanism that is involved in BRCA1 activation of the Gadd45 promoter, we have mapped the BRCA1-regulatory elements in the Gadd45 promoter. These BRCA1-responsive elements are localized at the region of the Gadd45 promoter between –107 to –57. Deletion of this region has been shown to abrogate BRCA1 activation of the Gadd45 promoter.

   Recently, we have mapped the BRCA1-responsive element in the Gadd45 promoters. This regulatory region contains two Oct-1 sites and one CAAT box. Deletion or mutations of these motifs have been shown to disrupt BRCA1 activation of the Gadd45 promoter.

2. To further confirm that Oct-1 and CAAT box sites play roles in the regulation of Gadd45 promoter by BRCA1, we made several constructs, where Oct-1 or CAAT box sites were put in the upstream of a minimal polyomaviral promoter, and found these construct that harboring Oct-1 or CAAT sites were able to be activated by BRCA1 expression or DNA damage. Taken together with our previous findings, these results have provided us with the direct evidence that BRCA1 activation of the Gadd45 gene is mediated through the Oct-1 and CAAT box motifs that localize in the Gadd45 promoter.

3. Additionally, we also demonstrated the associations of BRCA1 with Oct-1 and NF-YA transcription factors. Depletion of Oct1 and NF-YA proteins is able to disrupt the binding of BRCA1 protein to the Gadd45 promoter, indicating that the involvement of Oct-1 and NF-YA transcription factors in up-regulation of the Gadd45 promoter by BRCA1 protein.

4. Most recently, we have found that ATR kinase may be involved in the signaling pathway that mediate BRCA1 activation of the Gadd45 promoter. Wild-type ATR, but not mutant forms, might be able to phosphorylate Oct-1 protein and stabilize Oct-1. In addition, the phosphorylation of Oct-1 by ATR will increase the binding affinity of Oct1 to its specific motif and in turn enhance the transactivation of Oct-1 on its downstream genes.

3. KEY RESEARCH ACCOMPLISHMENTS

The key accomplishments of this project during the last year include: (1). Through the established Gadd45 inducible cell lines, we further demonstrated that Gadd45 is capable of mediating BRCA1's role in the control of cell cycle checkpoint and growth suppression. (2). Using Gadd45 deficient cells (both human line and MEFs from Gadd45 knowckouts), we demonstrated that BRCA1-induced cell cycle G2-M arrest and growth suppression is at least in part mediated through Gadd45 protein. (3). We have demonstrated that BRCA1 activation of the Gadd45 promoter is mediated via OCT-1 and CAAT motifs that localize on the Gadd45 promoter. Furthermore, BRCA1 activation of the Gadd45 promoter has been shown to require the interaction of BRCA1 with Oct-1 or NF-YA transcription factors. (4). We made first demonstration that ATR kinase is involved in the BRCA1 activation of the Gadd45 promoter. ATR kinase has been found to stabilize Oct-1 protein and enhance the Binding of Oct-1 to its specific motifs

4. REPORTABLE OUTCOMES

The reportable outcomes related to this project include:

(1). Two papers have been published in ONCOGENE (one as a full article, and one as short report), which is a prestigious journal in the cancer research field (see Appendix).

(2). In the retreat organized by the University of Pittsburgh Cancer Institute, the poster presentation from this project won the first-place award.

(3). Under the support from DOD, one postdoctoral fellow is hired to obtain training in my laboratory.

5. CONCLUSION

This DOD-funded project has been going very well during the third year. Although we have applied for a one-year no-cost extension, the findings in this project over the past three years have strongly demonstrated a novel pathway (BRCA1-Gadd45) in the control of cell cycle G2-M checkpoint, indicating that role of BRCA1 in maintenance of genomic stability may be mediated through Gadd45. Future work will be focused on further defining the detailed biochemical and molecular mechanism by which the pathway plays important role in preventing breast cancer. In addition, we recently found two BRCA1-assocated proteins, which might synergistically work together with Gadd45. Completion of this project will provide insight into development of new anti-breast cancer drugs.

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Appendices

1. **Reprint published in Oncogene (Jin et al, 2002)**
2. **Reprint in press in Oncogene (Jin at al, 2003)**

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GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity

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In response to DNA damage, the cell cycle checkpoint is an important biological event in maintaining genomic fidelity. Gadd45, a p53-regulated and DNA damage inducible protein, has recently been demonstrated to play a role in the G2-M checkpoint in response to DNA damage. In the current study, we further investigated the biochemical mechanism(s) involved in the *GADD45*-activated cell cycle G2-M arrest. Using the tetracycline-controlled system (tet-off), we established *GADD45*-inducible lines in HCT116 (wild-type p53) and Hela (inactivated p53 status) cells. Following inducible expression of the Gadd45 protein, cell growth was strongly suppressed in both HCT116 and Hela cells. Interestingly, HCT116 cells revealed a significant G2-M arrest but Hela cells failed to arrest at the G2-M phases, indicating that the *GADD45*-activated G2-M arrest requires normal p53 function. The *GADD45*-induced G2-M arrest was observed independent of p38 kinase activity. Importantly, induction of Gadd45 protein resulted in a reduction of nuclear cyclin B1 protein, whose nuclear localization is critical for the completion of G2-M transition. The reduced nuclear cyclin B1 levels correlated with inhibition of Cdc2/cyclin B1 kinase activity. Additionally, overexpression of cyclin B1 substantially abrogated the *GADD45*-induced cell growth suppression. Therefore, *GADD45* inhibition of Cdc2 kinase activity through alteration of cyclin B1 subcellular localization may be an essential step in the *GADD45*-induced cell cycle G2-M arrest and growth suppression. *Oncogene* (2002) **21,** 8696–8704. doi:10.1038/sj.onc.1206034

Keywords: p53; *GADD45*; G2-M arrest; cyclin B1

Introduction

In response to DNA damage, mammalian cells arrest at the transition from G1 to S phase (G1-S checkpoint) and G2 to M phase (G2-M checkpoint) (Hartwell and Weinert, 1989). Cell cycle arrest at these checkpoints prevents DNA replication and mitosis in the presence of DNA damage. Inactivation of those cell cycle checkpoints results in genomic instability, which is closely associated with cell transformation and tumorigenesis. In addition, disruption of normal cell cycle controlling machinery often has dramatic consequences on therapeutic sensitivity (Elledge, 1996; Hartwell and Kastan, 1994; Kohn *et al.*, 1994; O'Connor and Kohn, 1992; Paulovich *et al.*, 1997).

Currently, the mechanism(s) by which DNA damaging agents activate cell cycle G1-S checkpoint is well understood. The tumor suppressor p53 gene plays a critical role in the control of G1-S arrest. Following DNA damage, p53 transcriptionally up-regulates p21 (el-Deiry *et al.*, 1993), one of the p53-downstream genes and a potent cell cycle-dependent kinase inhibitor. Subsequently, induced p21 forms complexes with Cdk-cyclin and inhibits the activity of cdk4-cyclin D, Cdk6-cyclin D, Cdk2-cyclin E, and Cdk2-cyclin A, and in turn transiently arrest cells at the G1-S transition (Harper *et al.*, 1993; Sherr and Roberts, 1995; Xiong *et al.*, 1993; Zhang *et al.*, 1994). It has been demonstrated that the disruption of endogenous p21 abrogates the G1-S checkpoint after cell exposure to DNA damage (Waldman *et al.*, 1995). P53 has also been implicated in the control of the G2-M checkpoint. Introduction of p53 into p53-deficient human fibroblasts results in both G1-S and G2-M arrest (Agarwal *et al.*, 1995; Stewart *et al.*, 1995) and the HPV-16 E6 viral oncoprotein, which blocks p53 function, has been shown to decrease the stringency of the mitotic checkpoint (Thompson *et al.*, 1997). Recent evidence indicates that p53 and p21 are required for maintaining the G2 checkpoint in human HCT116 cells (Bunz *et al.*, 1998). In addition, 14-3-3, which blocks Cdc25 activity and arrests cells at the G2-M transition, is demonstrated as one of the p53 downstream genes

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(Hermeking *et al.*, 1997). Most recently, *GADD45*, a p53-regulated and DNA damage-inducible gene, is found to play an important role in the G2-M checkpoint in response to certain types of DNA damaging agents (Jin *et al.*, 2000; Wang *et al.*, 1999; Zhan *et al.*, 1999).

However, the G2-M checkpoint is complex and may involve redundant controls including both p53-independent and p53-dependent mechanisms. It has been well accepted that many of the G2-M regulators appear to ultimately target Cdc2, a protein kinase required for the mitotic entry in mammalian cells (Elledge, 1996; O'Connor, 1997). Activation of Cdc2 kinase requires its association with mitotic cyclins (cyclin B1 and cyclin A), and phosphorylation at Thr-161 and dephosphorylation at Thr-14/Tyr-15 cells (Ducommun *et al.*, 1991; Elledge, 1996; O'Connor, 1997). After DNA damage, several G2-M regulators, including Chk1, Chk2, 14-3-3 and ATM, alter Cdc2 activity by inhibiting dephosphorylation of Cdc25C phosphatase. The inhibition of Cdc25C activity prevents the removal of inhibitory phosphorylations from Thr-14 and Tyr-15 of Cdc2 (Elledge, 1996; O'Connor and Fan, 1996; Paulovich *et al.*, 1997). In addition, DNA damage is able to suppress Cdc2 activity by inhibiting the accumulation of cyclin B1 mRNA and protein (Bernhard *et al.*, 1995; Muschel *et al.*, 1991, 1992). Delayed entry into mitosis following DNA damage also correlates with nuclear exclusion of cyclin B1 protein (Toyoshima *et al.*, 1998).

The *GADD45* gene is induced by a variety of DNA damaging agents, including ionizing radiation (IR), methyl methansulfonate (MMS), UV radiation (UV), hydroxyurea and medium starvation (Fornace *et al.*, 1988, 1989; Papathanasiou *et al.*, 1991). The IR-induction of *GADD45* is transcriptionally regulated by p53 via a p53-binding site in the third intron (Kastan *et al.*, 1992; Zhan *et al.*, 1994a). In contrast, *GADD45* induction by UV radiation or MMS treatment is detected in all mammalian cells regardless of p53 status. However, recent evidence shows that p53 can still contribute to cellular responses to UV, MMS and medium starvation although it is not required (Zhan *et al.*, 1996, 1998). Gadd45 is a nuclear protein and binds to multiple important cellular proteins such as proliferating cell nuclear antigen (PCNA) (Hall *et al.*, 1995; Smith *et al.*, 1994), p21 protein (Chen *et al.*, 1996; Kearsey *et al.*, 1995; Zhao *et al.*, 2000), core histone protein (Carrier *et al.*, 1999), MTK/MEKK4 (Takekawa and Saito, 1998), an upstream activator of the JNK pathway, and Cdc2 protein kinase. The presence of Gadd45 in these complexes indicates that Gadd45 may be an important player in cell cycle control, DNA repair and the regulation of signaling pathway. The role of *GADD45* in maintaining genomic stability has been demonstrated by the recent finding that the mouse embryonic fibroblasts (MEF), derived from gadd45-null mice exhibit aneuploidy, chromosomal aberrations, gene amplification and centrosome amplification. Additionally, gadd45-knockout mice display increased radiation carcinogenesis (Hollander *et al.*, 1999). In this study, we have further investigated the role of *GADD45* in the G2-M checkpoint and demonstrated that the *GADD45*-induced G2-M arrest depends on normal cellular p53 function, but is independent of p38 kinase activity, which is reported to be required for the initiation of the G2-M checkpoint after UV radiation. In addition, inducible expression of Gadd45 protein has been shown to result in alterations of cyclin B1 subcellular distribution, which might be a consequence of the interaction of Gadd45 with Cdc2 proteins.

Results

Inducible expression of Gadd45 protein suppresses human cell growth

To further investigate the biological mechanism(s) by which *GADD45* plays a role in the control of cell cycle regulation, we established tetracycline-regulated *GADD45*-inducible cell lines in human cervical cancer Hela cells (see Materials and methods), where cellular p53 function is inactivated, and human colorectal carcinoma HCT116 cells, which has wild-type p53 and normal p53 function. As shown in Figure 1a, both Hela *GADD45*-inducible cells and HCT116 *GADD45*-inducible cells exhibited extremely low basal levels of the endogenous Gadd45 protein. Following withdrawal of tetracycline, Gadd45 protein was greatly induced and presented more than 10-fold induction in both cell lines. Next, the effect of Gadd45 protein on growth suppression was examined in these two *GADD45*-inducible cell lines. To perform this experiment, 500, 1000 or 2000 cells were seeded and grown in DMEM medium at 100-cm dishes 16 h prior to tetracycline withdrawal. After removing tetracycline, cells continued to grow for 14 days and then were fixed, scored for colonies containing more than 50 cells. Similar to our previous finding that overexpression of *GADD45* protein via transient transfection inhibits tumor cell growth (Zhan *et al.*, 1994b), inducible expression of Gadd45 protein in both Hela and HCT116 lines strongly suppressed colony formation (Figure. 1B), indicating a suppressive role of *GADD45* in cell growth regardless of p53 status. In agreement with this observation, both Hela and HCT116 lines with inducible expression of Gadd45 protein exhibited a substantial slow growth rate (results not shown). Taken together, these results indicate that *GADD45* plays a negative role in the control of cell progression.

GADD45 induced cell cycle G2-M arrest depends on normal cellular p53 function

In order to further determine the role of *GADD45* in the control of cell cycle G2-M arrest, cell cycle distribution analyses were conducted in both HCT116 and Hela *GADD45*-inducible cell lines. Following removal of tetracycline, *GADD45*-inducible cells were collected at 24 h or 36 h and subject to flow cytometry

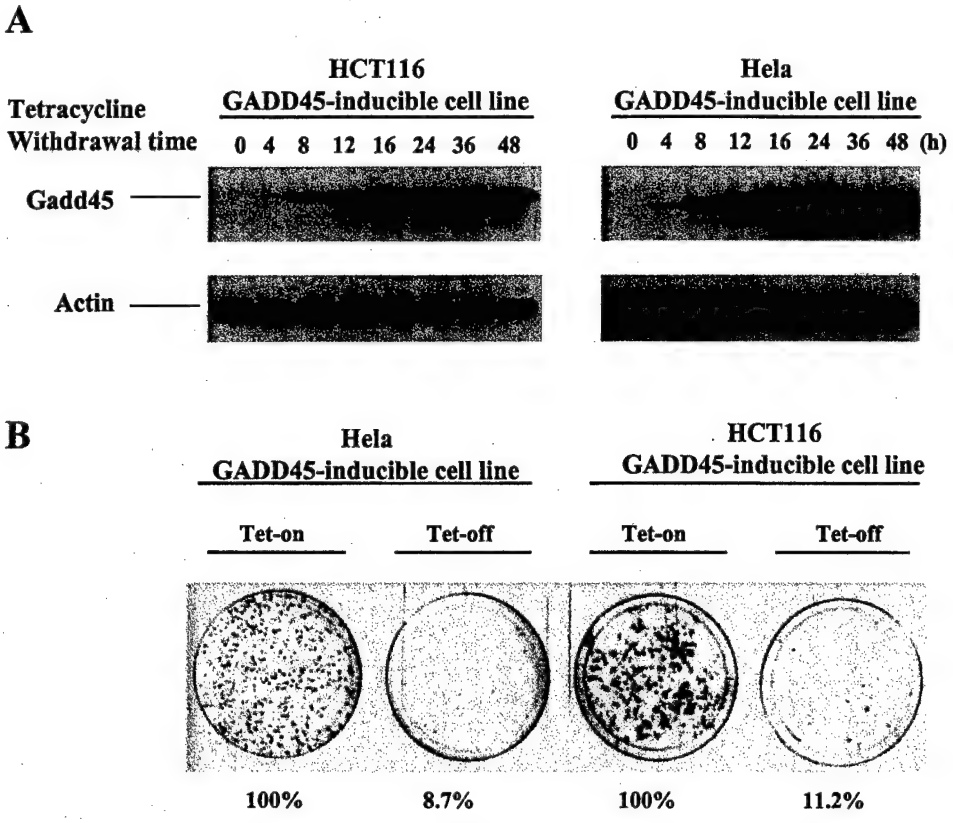

Figure 1 Tumor cell growth suppression by Gadd45 protein. (**a**) Induction of Gadd45 protein in HCT116 and Hela cells controlled by the Tet-off system. HCT116 *GADD45*-inducible lines and Hela *GADD45*-inducible cell lines were established as described in Materials and methods. Cells were placed in 100 mm dishes at a density of 4×10^5 and grown in DMEM medium containing tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time points for preparation of cellular protein. 100 μg of whole cell protein was used for immunoblotting analysis with anti-*GADD45* antibody. As a loading control, anti actin antibody was included. (**b**) Induction of Gadd45 protein suppresses cell growth. HCT116 and Hela *GADD45*-inducible cells were seeded at a density of 1000 cells per 100 mm dish and grown in medium containing 2 μg/ml of tetracycline. After 16 h, medium was removed and plates were washed three times with PBS, then fresh medium containing no tetracycline was added into plates. The cells were fixed and stained at 14 days and scored for colonies containing at least 50 cells. The experiments were performed four times and only representative results were shown here

analysis. The results presented in Figure 2 depict a representative profile of cell cycle distribution in those cells. In the HCT116 *GADD45*-inducible line (wt p53 status), cells grown in the presence of tetracycline presented 14–16% population in G2-M phase. However, inducible expression of Gadd45 protein resulted in a clear accumulation of the G2-M fraction. Approximately 32% of the cells were arrested at the G2-M phase of the cell cycle in the absence of tetracycline, indicating that *GADD45* expression alone is able to halt cells in G2-M phase. In contrast, after inducible expression of Gadd45, Hela cells (inactivated p53 status) did not exhibit any evident changes of cell cycle distribution. In consistence with these results, introduction of *GADD45* expression vector into HCT116 via transient transfection resulted in increased G2-M population in HCT116 but not in HCT116 p53−/−, where p53 alleles were knocked out by homologous recombination approach (result not shown). These observations further demonstrate that *GADD45*-mediated G2-M arrest requires normal cellular p53 function.

GADD45 induced cell cycle G2-M arrest is not affected by inhibitors of p38

The mitogen-activated protein kinase p38 has recently been reported to play a critical role in cell cycle G2-M checkpoint in response to UV radiation (Bulavin *et al.*, 2001). To understand whether p38 kinase activation contributes to the *GADD45*-induced G2-M arrest, the mitotic index was measured in *GADD45* inducible cells in the presence of p38 kinase inhibitor, SB202190. In Figure 3a, high mitotic indices were observed in HCT116 cells treated with nocodazole. In response to UV radiation, mitotic indices substantially decreased, indicating that UV treatment arrests cells in the G-M transition. Addition of p38 inhibitor SB202190 at a concentration of 10 μM was shown to greatly attenuate the UV-induced G2-M arrest. In Figure 3b, inducible expression of Gadd45 protein exhibited low mitotic indices, which reflects a significant G2-M arrest by Gadd45. However, p38 inhibitor SB202190 (10 μM) showed little effect on the Gadd45-induced G2-M arrest. These results suggest that the *GADD45*

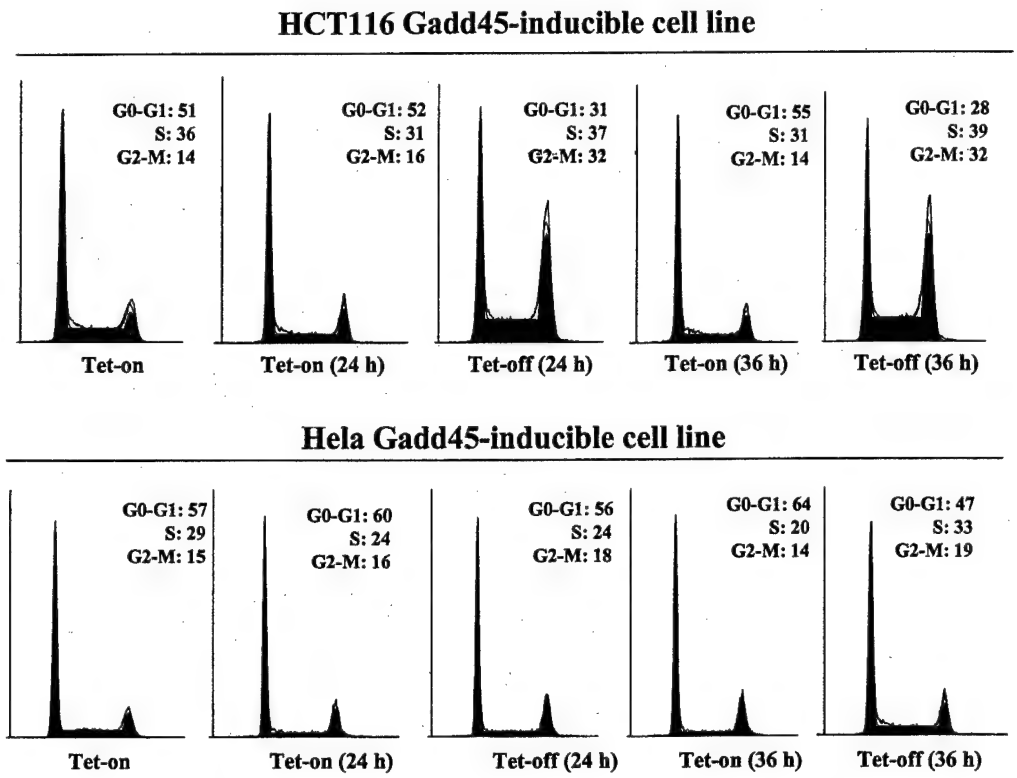

Figure 2 Cell cycle G2-M arrest following inducible expression of Gadd45 protein in both HCT116 and Hela cells. HCT116 and Hela *GADD45*-inducible cells were grown in DMEM medium with 10% fetal bovine serum in the presence of tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time points and subject to flow cytometric analysis as described in Materials and methods

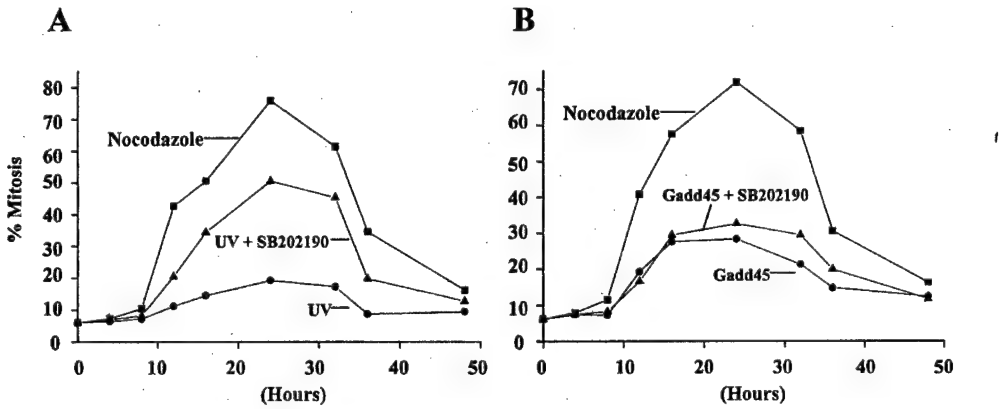

Figure 3 Mitotic entry after UV radiation or inducible expression of Gadd45 protein. (**a**) HCT116 cells were UV irradiated in the presence of 10 μM p38 kinase inhibitor SB203580 and mitotic indices were determined as described in Materials and methods. (**b**) HCT116 *GADD45*-inducible cells were grown in medium with tetracycline (2 μg/ml). Upon the withdrawal of tetracycline, cells were exposed to 10 μM p38 kinase inhibitor SB203580 and followed by determination of mitotic indices at the indicated time points

induction of cell cycle G2-M checkpoint does not require activation of p38 kinase.

Expression of Gadd45 protein alters the level of nuclear cyclin B1 but does not affect phosphorylation statuses of Cdc25C or Chk1

In our previous report, we have demonstrated that Gadd45 protein physically interacts with Cdc2 kinase, dissociates Cdc2/cyclin B1 complexes and in turn inhibits Cdc2 kinase activity, but does not alter Cdc2 phosphorylation status (Zhan *et al.*, 1999). However, the biochemical consequence of the interaction between Gadd45 and Cdc2 remains to be further defined. Since nuclear localization of cyclin B1 protein is thought to be critical for the completion of G2-M transition, we further examined cyclin B1 protein distributions in both the nucleus and cytoplasm. As shown in Figure

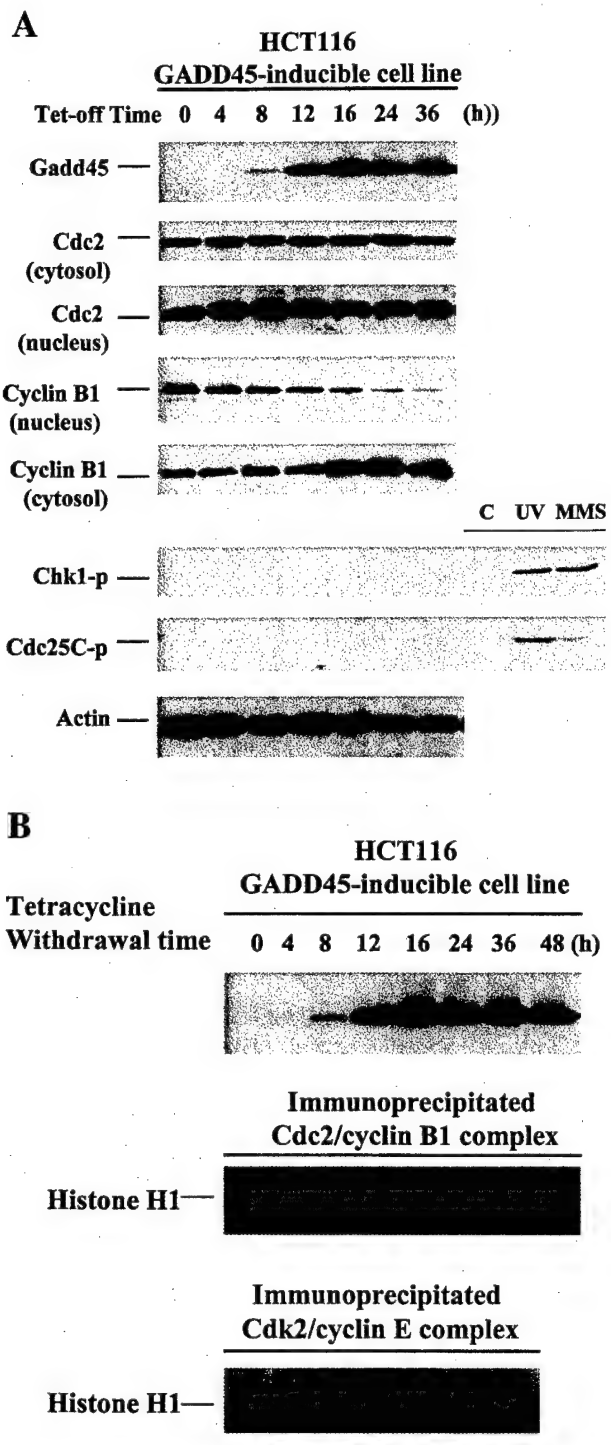

Figure 4 Subcellular localization of cyclin B1 protein and inhibition of Cdc2 kinase activity following inducible expression of Gadd45. (**a**) Cellular proteins were prepared from HCT116 *GADD45*-inducible cells after withdrawal of tetracycline at the indicated time points. 100 μg of proteins were loaded onto SDS–PAGE gel for detection of subcellular distribution of cyclin B1 protein and phosphorylations of CHK1 or Cdc25C. (**b**) One mg of cellular proteins isolated from HCT116 *GADD45*-inducible cells at the indicated time points was immunoprecipitated with anti-cyclin B1 or cyclin E antibodies, and histone H1 kinase assays were performed as described in Materials and methods. Labeled histone H1 was detected by autoradiography following size separation on a SDS–PAGE gel

4a, following inducible expression of Gadd45 protein in HCT116 cells, there were no evident alterations of Cdc2 protein in both the cytosol and nuclear compartments. Interestingly, nuclear cyclin B1 protein exhibited a significant reduction in response to induction of Gadd45. In support of this observation, cytosol cyclin B1 appeared to increase after Gadd45 induction. These results indicate that Gadd45 induction caused subcellular redistribution of cyclin B1 protein. In the same experiment, Chk1 and Cdc25C phosphorylation statuses were also examined, but no phosphorylations of Chk2 or Cdc25C were detected following Gadd45 induction. However, cells treated with UV and MMS displayed increased phosphorylations for Chk2 and Cdc25C. Additionally, we analysed Cdc2 and Cdk2 kinase activity following Gadd45 protein expression and found Cdc2 was inhibited by Gadd45 but Cdk2 kinase activity remained at the similar levels after Gadd45 expression (Figure 4b). Taken together, Gadd45 protein is able to alter cyclin B1 nuclear localization and in turn inhibits Cdc2 kinase activity.

Cyclin B1 abrogates the GADD45-induced cell growth suppression

We have previously demonstrated that *GADD45*-induced growth suppression (Zhan *et al.*, 1994b), in a great content, correlates with its inhibition of Cdc2/cyclin B1 kinase activity. It is assumed that interaction of Gadd45 with Cdc2 causes dissociation of the Cdc2/cyclin B1 complex, and in turn alters subcellular localization of cyclin B1, which contributes to the loss of Cdc2 kinase activity. Therefore, we examined whether introduction of cyclin B1 into cells can rescue *GADD45*-induced growth-suppression. To do this, a *GADD45* expression vector was co-transfected with expression vectors for cyclin B1, Cdc2, or cyclin D1 into HCT116 cells (p53 wt line). In Figure 5, expression of *GADD45* in HCT116 cells

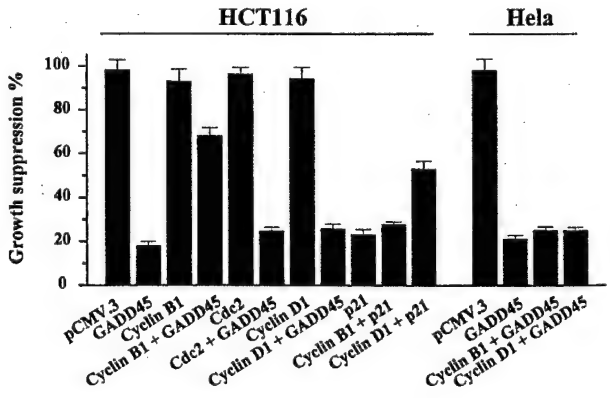

Figure 5 Effect of cyclin B1 expression on the *GADD45*-induced cell growth suppression. Human colorectal carcinoma HCT116 cells were transfected with the indicated expression vectors. Following selection with G418 for 2 weeks, cells were fixed and the colonies that contained at least 50 cells were counted. Quantitative results represent the average of three individual experiments

suppressed 80% cell growth. While in the presence of cyclin B1 overexpression, *GADD45* only generated 30% growth suppression, indicating that cyclin B1 is able to abrogate *GADD45*-induced growth inhibition. In contrast, overexpression of both Cdc2 and cyclin D1 failed to rescue the *GADD45*-induced growth suppression. Additionally, cyclin B1 had little effect on p21, suggesting that p21-induced cell growth might not be mainly through its role in the G2-M arrest. Interestingly, *GADD45* was able to suppress cell growth in Hela cells, which contains inactivated p53 and does not exhibit *GADD45*-induced G2-M arrest. However, both cyclin B1 and cyclin D1 were incapable of abrogating *GADD45*-induced cell growth, indicating *GADD45* inhibits cell growth in cells with abnormal p53 probably through a different mechanism distinct from the inhibition of Cdc2 kinase activity in p53 wt cell lines.

Discussion

In this report, we further investigated the role of Gadd45, a p53-regulated and stress-inducible protein, in the control of cell cycle G2-M checkpoint. Using tetracycline-controlling system (tet-off), we established *GADD45*-inducible lines in both HCT116 (wt p53) and Hela (negative p53 status) cells. Therefore, induction of Gadd45 protein was nicely manipulated by the withdrawal of tetracycline. Following inducible expression of Gadd45, cell growth was strongly inhibited in both HCT116 and Hela lines. In consistence with our previous finding that the introduction of *GADD45* expression vector into human normal fibroblast via microinjection approach causes cells to arrest at the G2-M transition, induction of Gadd45 protein in the HCT116 *GADD45*-inducible line greatly increased cell population in G2-M phase, but Gadd45 expression was unable to induce G2-M arrest in Hela cells, which contain inactivated p53. The *GADD45*-induced G2-M arrest appeared independent of p38 kinase activity, as employment of p38 kinase inhibitor (SB202190) did not abrogate GADD-induced G2-M arrest. More importantly, overexpression of Gadd45 protein was shown to result in reduction of nuclear cyclin B1 protein and inhibited Cdc2 kinase activity, but had no effect on Chk1, Cdc25C phosphorylation and Cdk2 activity. In addition, co-introduction of cyclin B1 expression vector was able to substantially disrupt the *GADD45*-induced growth suppression.

The tumor suppressor p53 gene has been implicated in the control of cell cycle checkpoint in response to genotoxic stress (Bunz *et al*., 1998, 1999; Kastan *et al*., 1991, 1992). The role for p53 in G1-S arrest is clearly shown to be mediated though p21 (Harper *et al*., 1993; Sherr and Roberts, 1995; Xiong *et al*., 1993; Zhang *et al*., 1994). However, the role of p53 in the control of the G2-M arrest is under debate and remains to be further elucidated. It is postulated that as one of the p53-targeted genes (Kastan *et al*., 1992; Zhan *et al*., 1994a), *GADD45* might be a strong player in mediating p53-regulated cell cycle G2-M checkpoint. Previous studies have shown that Gadd45 protein interacts with Cdc2 and dissociates the Cdc2/cyclin B1 complex (Jin *et al*., 2000; Zhan *et al*., 1999). Subsequently, 'free' cyclin B1 protein dissociated from the Cdc2 complex is more likely pumped out from the nucleus, probably by the nuclear transport system. As a result of exclusion of cyclin B1 protein from the nucleus, Cdc2 kinase activity is inhibited and followed up by the cell cycle G2-M arrest. This goes along with the finding by Toyoshima *et al*. (1998) that DNA damage causes increased nuclear export of cyclin B1 and in turn arrests cells at the G2-M transition. Our observations that inducible expression of *GADD45* protein alters cyclin B1 nuclear localization (Figure 4) have suggested that exclusion of nuclear cyclin B1 protein by Gadd45 might be an essential step for the *GADD45*-induced G2-M arrest. Therefore, the findings in this work have further presented the precise evidence that the p53-*GADD45* pathway is well involved in the control of G2-M arrest.

The mechanism(s) for p53 dependence of the *GADD45*-induced cell cycle G2-M arrest is not clear at the present time. Bunz *et al*. (1998) has reported that cells with disrupted p53 display an impaired G2-M checkpoint after DNA damage, and suggested that the role for p53 in sustaining G2-M arrest after DNA damage might be mediated through p21. However, our previous investigations have already demonstrated that p21 is not required for *GADD45*-induced G2-M arrest, since introduction of *GADD45* expression vector into p21 deficient cells, where endogenous p21 has been disrupted, is able to generate G2-M arrest (Wang *et al*., 1999). We have also not found any alterations of MDM2 protein level following Gadd45 induction and no physical interactions between Gadd45 and MDM2 proteins (result not shown). Therefore, both p21 and MDM2 appear not to be the candidates to mediate the role for p53 in *GADD45*-induced G2-M arrest. Future investigation is required to explore the mechanism by which p53 is required for the *GADD45*-induced G2-M arrest.

The mitogen-activated kinase p38 is required for initiating the G2-M checkpoint after UV radiation, probably through phosphorylating Cdc25B at serines 309 and 361 (Bulavin *et al*., 2001). However, the *GADD45*-induced G2-M arrest is independent of p38 kinase activity. These results have further confirmed that Gadd45 acts at the late G2-M transition or early mitotic phase, instead of at the initiation of G2-M transition. In addition, the inhibitory effect of the Gadd45 protein appears to be specifically localized on Cdc2/cyclin B1 complex, as induction of Gadd45 protein does not alter phosphorylations of Chk1 and Cdc25C. Overexpression of cyclin B1 protein has been found in certain types of human tumors although the biological function of this overexpressed protein in tumorigenesis remains unclear (Soria *et al*., 2000). Interestingly, Overexpression of cyclin B1 is closely associated with loss of a p53 function (Yu *et al*., 2002). In Figure 5, co-expression of cyclin B1 with Gadd45

protein abrogated the Gadd45-induced cell growth suppression. This evidence has provided a new insight into understanding on the role of cyclin B1 in development of genomic instability and tumorigenesis.

GADD45 was shown to suppress cell growth in both HCT116 (wt p53) and Hela (inactivated p53) cells, regardless of p53 status (Figure 5). However, *GADD45* only generated G2-M arrest in HCT116 cells, but not in Hela cells, suggesting that the *GADD45*-induced growth suppression is complex and might involve the biological events distinct from the G2-M arrest. In fact, Takekawa and Saito (1998) have previously reported that *GADD45* interacts with MTK1/MEKK4, an upstream activator of the JNK pathways, and induced apoptosis in Hela cells. Therefore, *GADD45* is able to play a negative role in cell growth probably through both cell cycle arrest and apoptosis. The importance of *GADD45* in maintenance of genomic fidelity has been presented by the evidence that gadd45-null mice generated by gene targeting exhibit aneuploidy, chromosome aberrations, gene amplification and centrosome amplification, and increased tumorigenesis after DNA damaging agents (Hollander *et al*., 1999). Therefore, the current studies have further demonstrated the mechanism(s) by which *GADD45* plays a role in maintaining genomic stability and provides insight into understanding the p53-*GADD45* pathway in cellular response to genotoxic stress.

Materials and methods

Establishment of the GADD45 inducible cell line and cell culture

To establish *GADD45* tet-off inducible cell lines, human colorectal carcinoma HCT116 cells were initially transfected with pTet-Off plasmid (Clontech, Palo Alto, CA, USA), which is commercially available and expresses the tTA regulator proteins, and the G418-resistant colonies were selected and amplified. Next, the cells expressing tTA proteins were subject to second round transfection with pTRE–*GADD45* construct, where the *GADD45* gene was inserted into *Bam*H1/*Hind*III sites of pTRE plasmid (Clontech). The cells transfected with pTRE-*GADD45* plasmid were selected by hygromycin at 200 μg/ml for 14 days, and each hygro-resistant colony was separately collected for detection of Gadd45 protein expression under tet-off system. In the case of Hela *GADD45* inducible lines, Hela cells expressing tTA were commercially obtained from Clontech and transfected with pTRE-*GADD45* construct and the hygro-resistant cells were selected as described in HCT116 cells. *GADD45*-inducible cells were grown in DMEM medium supplemented with 10% fetal bovine in the presence of tetracycline at a concentration of 2 μg/ml. To induce expression of Gadd45 protein, DMEM containing tetracycline was removed and the plates were washed four times with PBS, and fresh DMEM medium containing no tetracycline was then added to cells. Cells were collected at the indicated time points for examination of induced Gadd45 protein.

Antibodies and immunoblotting analysis

The following antibodies were used in the experiments; *GADD45*, Cdc2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin B1 (Pharmingen, San Diego, CA, USA) and Cdc25C and Chk1 phosphorylation-sites specific antibodies (Cell Signaling Technology Inc, Beverly, MA, USA). *GADD45*-inducible cells were exponentially grown in DMEM medium containing tetracycline at a concentration of 2 μg/ml. After withdrawal of tetracycline, cells were collected at the indicated time. For preparation of cellular protein, plates were rinsed with PBS and cells were lysed in PBS containing100 μg/ml phenyl-methylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin and 1% NP-40 (lysis buffer). Lysates were collected by scraping and cleared by centrifugation at 4°C. 100 μg of cellular protein was loaded onto 12% SDS–PAGE gel and transferred to Protran membranes. Membranes were blocked for 1 h at room temperature in 5% milk, washed with PBST (PBS with 0.1% Tween-20), and incubated with indicated antibodies for 2 h. Membranes were washed four times in PBST and HRP-conjugated anti-mouse antibody was added at 1:4000 in 5% milk. After 1 h, membranes were washed and detected by ECL (Amersham, Arlington Height, IL, USA) and exposed to X-ray film (Kodak, Rochester, NY, USA).

Growth suppression assay

Five hundred, 1000, or 2000 cells from Hela or HCT116 *GADD45*-inducible lines were seeded in 100-cm dishes and grown in DMEM medium containing 2 μg/ml tetracycline for 16 h. Following withdrawal of tetracycline, cells were fixed at 14 days and scored for colonies containing at least 50 cells (Zhan *et al*., 1994b).

Cdc2 and Cdk2 kinase assays

Cellular lysates isolated from the *GADD45*-inducible cells were incubated with 10 μl of cyclin B1 antibody (Pharmingen) or 20 μl of Cdk2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 20 μl of protein A/G agarose beads (Santa Cruz Biotechnology) at 4°C for 6 h. Immunocomplexes were washed four times with lysis buffer and followed by kinase buffer. Histone H1 kinase assays were then performed in the presence of 10 μg of histone H1 (Upstate Biotechnology, Lake Placid, NY, USA), 15 mM $MgCl_2$, 7 mM β-glycerol phosphate, 1.5 mM EDTA, 0.25 mM sodium orthvanadate, 0.25 mM DTT and 10 μCi of γ-^{32}P ATP in 30 μl volume. After 15 min at 30°C, the reactions were mixed with an equal amount of standard 2×SDS protein denature loading buffer, sized-separated on a 12% SDS–PAGE gel (Zhan *et al*., 1999).

Flow cytometry analysis

HCT116 and Hela *GADD45*-inducible cells were plated into 100-mm dishes at a density of 6×10^5 and grown in DMEM containing 2 μg/ml of tetracycline. Sixteen hours later, medium was removed and plates were washed four times followed by addition of fresh medium. After incubation for 36 h, cells were collected, washed with PBS, fixed with 70% ethanol for 2 h at 4°C. Cells were then incubated with RNase (10 μg/ml) for 30 min and stained with propidium iodine (Sigma; 50 μg/ml). Cell cycle analysis was performed using Becton Dickson fluorescence-activated cell analyzer. At least 10,000 FITC positive cells were analysed using CellQuest and Modfit programs (Wang *et al*., 1999).

Analysis of mitotic index in HCT116 GADD45-inducible cells

HCT116 *GADD45*-inducible cells were seeded at a density of 6×10^5 in DMEM containing 2 μg/ml of tetracycline.

Following withdrawal of tetracycline, cells were grown in the presence of 10 μM p38 kinase inhibitor SB203580 and harvested at the indicated time points, fixed in methanol: acetic acid (3:1), spread on glass microscope slides, air-dried and stained with 5% Giemsa. Nuclei exhibiting condensed, evenly staining chromosomes were scored as mitotic. At least 1000 cells were counted in each determination. Meanwhile, HCT116 cells treated with p38 kinase inhibitor SB203580 were exposed to UV radiation and subjected to analysis of mitotic index.

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SHORT REPORT

Gadd45a contributes to p53 stabilization in response to DNA damage

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p53 is an important molecule in cellular response to DNA damage. After genotoxic stress, p53 protein stabilizes transiently and accumulates in the nucleus, where it functions as a transcription factor and upregulates multiple downstream-targeted genes, including $p21^{Waf1/Cip1}$, Gadd45a and Bax. However, regulation of p53 stabilization is complex and may mainly involve post-translational modification of p53, such as phosphorylation and acetylation. Using mouse embryonic fibroblasts (MEFs) derived from Gadd45a knockouts, we found that disruption of Gadd45a greatly abolished p53 protein stabilization following UVB treatment. Phosphorylation of p53 at Ser-15 was substantially reduced in Gadd45a−/− MEFs. In addition, p53 induction by UVB was shown to be greatly abrogated in the presence of p38 kinase inhibitor, but not c-Jun N-terminal kinase (JNK) and extracellular-signal regulated kinase (ERK), suggesting that p38 protein kinase is involved in the regulation of p53 induction. Along with the findings presented above, inducible expression of Gadd45a enhanced p53 accumulation after cell exposure to UVB. Taken together, the current study demonstrates that Gadd45a, a conventional downstream gene of p53, may play a role as an upstream effector in p53 stabilization following DNA damage, and thus has defined a positive feedback signal in the activation of the p53 pathway.
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Keywords: p53; Gadd45a; DNA damage

Tumor suppressor p53 gene is the most frequently mutated gene (Vogelstein and Kinzler, 1992) and has been implicated in playing an important role in maintaining genomic fidelity by controlling cell cycle checkpoints and apoptotic process following cell exposure to genotoxic stress (Kastan *et al.*, 1992; Zhan *et al.*, 1994b; Miyashita and Reed, 1995; Waldman *et al.*, 1995; White, 1996; Levine, 1997). Following DNA damage, including ionizing radiation (IR), UV radiation (UV) and a variety of DNA alkylating agents, p53 protein is stabilized and activated as a transcription factor (Kastan *et al.*, 1991; Zhan *et al.*, 1993). Activated p53 transcriptionally upregulates its downstream genes, such as $p21^{Waf1/Cip1}$ (el-Deiry *et al.*, 1993), Gadd45a (Kastan *et al.*, 1992; Zhan *et al.*, 1994a; Zhan, 1998 #176) and Bax (Zhan *et al.*, 1994b; Miyashita and Reed, 1995), whose products are able to participate directly in the control of cell cycle arrest and apoptosis. Deregulation of p53 function in the cellular response to DNA damage results in genomic instability, which is closely associated with cell transformation and tumorigenesis.

Induction of p53 protein is the key step in the activation of p53-mediated signaling pathways (Kastan *et al.*, 1992). However, the regulation of p53 stabilization after genotoxic stress is complex and it is thought to occur primarily through a post-transcriptional mechanism as there are no changes in p53 mRNA levels following cell exposure to DNA damage (Kastan *et al.*, 1991). In nonstressed circumstances, p53 is degraded via the MDM2-mediated ubiquitin pathway (Haupt *et al.*, 1997; Honda *et al.*, 1997; Fuchs *et al.*, 1998a). MDM2 is also a p53-regulated protein and able to bind to p53, promoting p53 degradation in order to limit the length of p53 function of negatively regulating cell cycle progression (Wu *et al.*, 1993; Chen *et al.*, 1994). Therefore, one of the mechanisms by which p53 becomes stabilized may be through the inhibition of MDM2 binding to p53 protein. Previous studies reported that post-translational modification of p53 protein after DNA damage is able to remove the MDM2 inhibition of p53, block MDM2-mediated degradation of p53 and in turn cause accumulation of p53 (Shieh *et al.*, 1997; Ashcroft *et al.*, 1999; Oren, 1999). There are multiple phosphorylation sites at p53 protein and these sites are subjected to phosphorylation following genotoxic stress. Although various genotoxic stresses are able to stabilize p53 protein, different types of DNA-damaging agents act on varied phosphorylation sites of p53. For example, IR treatment results in p53 phosphorylation at Ser-15, Thr-18, Ser-20 and Ser-33 (Siliciano *et al.*, 1997; Dumaz and Meek, 1999; Shieh *et al.*, 1999; Unger *et al.*, 1999). UV radiation causes p53 phosphorylation at nearly every Ser site, including Ser-15, Ser-20, Ser-33, Ser-37 and Ser-46 (Siliciano *et al.*, 1997; Sakaguchi *et al.*, 1998; Bulavin *et al.*, 1999). In addition, a number of investigations have shown that p53 phosphorylation is carried out by a variety of

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upstream kinases, such as ATM, ATR, p38, c-Jun N-terminal kinase (JNK) and Casein kinase II (Milne *et al.*, 1992; Banin *et al.*, 1998; Canman *et al.*, 1998; Canman and Lim, 1998; Fuchs, 1998 #496; Bulavin *et al.*, 1999). These observations suggest that multiple signaling pathways are involved in p53 stabilization following cell exposure to various genotoxic stresses.

Gadd45a is a genotoxic stress-responsive gene and induced by a wide spectrum of DNA-damaging agents including IR, UV and many alkylating agents (Fornace *et al.*, 1988; Hollander *et al.*, 1993). Following DNA damage, Gadd45a induction is rapid, sensitive and transient (Zhan *et al.*, 1994a). Recent findings demonstrate that Gadd45a protein interacts with PCNA (Smith *et al.*, 1994), p21 (Kearsey *et al.*, 1995; Zhao *et al.*, 2000), Cdc2 (Zhan *et al.*, 1999; Jin *et al.*, 2000), MTK1/MEKK4 (Takekawa and Saito, 1998), and has been implicated in the control of cell cycle G2-M arrest (Wang *et al.*, 1999) as well as the maintenance of genomic stability. In agreement with these observations, Gadd45a knockout mice exhibit severe genomic instability and increased tumorigenesis by treatment with IR and UV radiation (Hollander *et al.*, 1999; Hildesheim *et al.*, 2002). Regulation of Gadd45a induction after DNA damage is complex and may involve both p53-dependent and -independent signaling pathways. It has been reported that Gadd45a induction by IR strictly depends on normal cellular p53 function, but Gadd45a induction by UV and MMS does not require p53 although p53 may contribute to the non-IR response of Gadd45a (Kastan *et al.*, 1992; Zhan *et al.*, 1996).

In the current study, we report that Gadd45a plays an important role in stabilization of p53 protein following UV radiation. As shown in Figure 1a, both E1A/ras-transformed normal mouse embryonic fibroblasts (MEFs, ER-1) and E1A/ras-transformed Gadd45aa−/− MEFs (ER-11) were treated with UVB radiation at the indicated doses (range from 0 to 400 J/m^2). At 8 h post-treatment, MEF cells were collected and prepared for the cellular protein as described previously. Whole-cell lysates (100 μg) were loaded onto 10% SDS–PAGE gels and measurement of p53 protein was performed with anti-mouse p53 antibody (Pharmigen, San Diego, CA, USA). In addition, actin protein was also measured as a loading control. The results in Figure 1a showed that UVB at doses of 10 or 30 J/m^2 did not significantly induce p53 in ER-1 cells, but an evident induction of p53 protein was observed in the cells treated with 100 J/m^2 of UVB. The maximal induction of p53 occurred in ER-1 cells exposed to the doses between 200 and 400 J/m^2. However, when ER-11 cells (Gadd45a−/− MEFs) were treated with UVB at the same doses (from 200 to 400 J/m^2), p53 induction was substantially weaker compared to that seen in ER-1. Next, we exposed both ER-1 and ER-11 to UVB radiation at a dose of 200 J/m^2 and detected p53 expression at the indicated time (0, 1, 2, 4, 6, 8 and 10 h). In ER-1 cells, a strong induction of p53 protein (more than sixfold) was clearly seen at the 2 h time point and maintained at a plateau till 10 h post-treatment. In contrast, ER-11 cells treated with 200 J/m^2 of UVB revealed a weaker induction of p53 protein

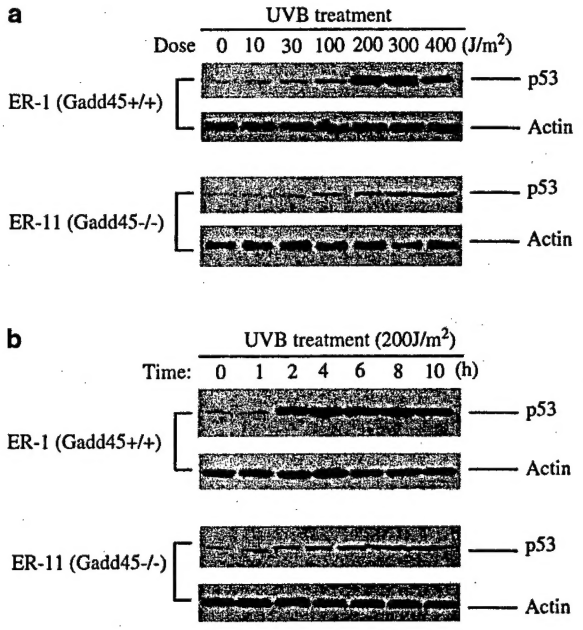

Figure 1 Attenuated induction of p53 protein in Gadd45a−/− cells treated with UVB. (**a**) Both E1A/ras-transformed normal MEFs (ER-1) and Gadd45a−/− MEFs (ER-11) were treated with UVB at different doses (range from 0 to 400 J/m^2). Cells were harvested at 8 h post-treatment and cellular proteins were prepared as described previously. Total cell protein (100 g) was loaded onto 10% SDS– PAGE gels. Following electrophoresis, the proteins were transferred to Immobilon membranes. The membranes were then blocked for 30 min in 5% milk at room temperature. Measurement of p53 protein was performed with anti-mouse p53 antibody (Santa Cruz Biotech, CA, USA). Immunoreaction was revealed using chemiluminescence detection procedure. As a loading control, detection of actin protein was included. Only visualized bands are shown; their estimated sizes were 53 kDa for p53 protein and 43 kDa for actin. All the experiments were performed at least three times. The results of the representative experiment are shown in this figure. (**b**) ER-1 and ER11 cells were exposed to UVB at a dose of 200 J/m^2 and harvested at the different time points. Detection of p53 expression was performed as described in (**a**)

(approximately twofold) at the same time points. These results indicate that Gadd45a has some role in the regulation of p53 induction after UVB treatment.

It has been well accepted that induction of p53 protein after DNA damage is mainly through a post-transcriptional mechanism. The post-translational modification of p53 at multiple phosphorylation sites greatly contributes to its stabilization following various genotoxic stresses (Shieh *et al.*, 1997; Ashcroft *et al.*, 1999; Oren, 1999). Among these phosphorylation sites, Ser-15 is critical for p53 stabilization after IR and UV (Lakin *et al.*, 1999; Tibbetts *et al.*, 1999). Therefore, we detected phosphorylation of p53 at Ser-15 using a phosphorylation-site-specific antibody (Santa Cruz Biotech, CA, USA). In Figure 2a, p53 phosphorylation at Ser-15 in ER-1 cells (Gadd45a+/+) treated with UVB was analysed. Cells were exposed to UVB at a range from 0 to 400 J/m^2 and cultures were harvested 4 h after treatment. Evidently, phosphorylation of Ser-15 in ER-1 cells was strongly induced by the exposure of the cells to

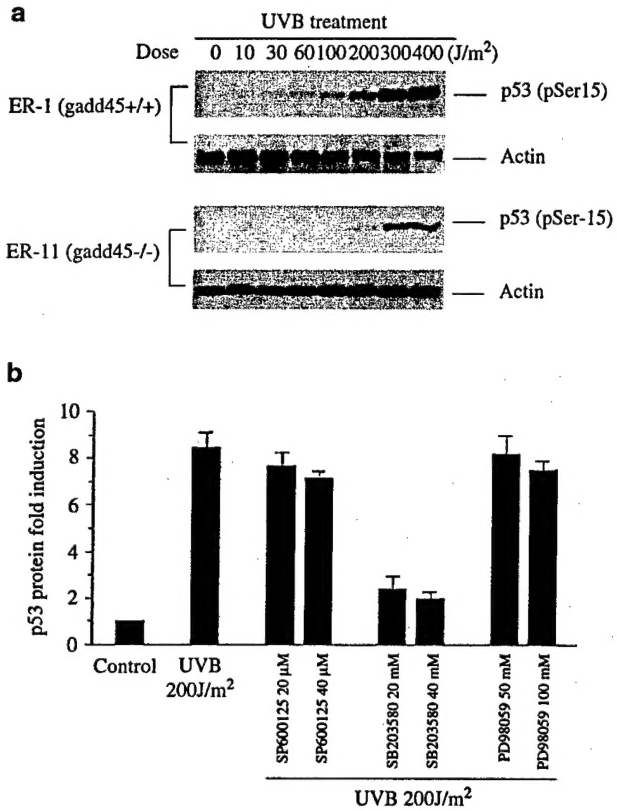

Figure 2 (**a**) Phosphorylation of p53 at Ser-15 in response to UVB treatment. Both ER-1 and ER-11 were treated with UVB radiation with different doses. Whole-cell protein was prepared and analysed for phosphorylation of p53 protein at Ser-15 using a phosphorylation-site-specific antibody. (**b**) ER-1 (E1A/ras-transformed normal MEF cells) were grown in the presence of kinase inhibitors for JNK (SP600125), p38 (SB203580) and ERK (PD98059) at the indicated concentration and treated with UVB at 200 J/m². Cellular proteins were prepared at 8 h post-treatment. Proteins (100 g) were loaded onto 10% SDS– PAGEgel for detection of cellular p53

UVB at doses between 200 and 400 J/m². Meanwhile, phosphorylation of Ser-15 in ER-11 cells exhibited less than half the level of that seen in ER-1 cells, indicating that disruption of Gadd45a results in decreased phosphorylation of Ser-15 for p53 protein following DNA damage. MAP kinase pathways, which include the extracellular-signal regulated kinase (ERK), stress-activated protein kinase-1/ JNK and stress-activated protein kinase-2/p38α and β (herein called p38), are found to be involved in post-translational modification of p53 protein. All ERK, JNK and p38 kinases are reported to phosphorylate p53 protein at various sites and may play roles in the stabilization of p53 in response to genotoxic stress (Fuchs *et al.*, 1998b; Bulavin *et al.*, 1999; Sablina *et al.*, 2001; Wang and Shi, 2001). To examine which MAP kinase pathways directly contributes to UVB-induced p53 induction, we treated ER-1 cells with 200 J/m² UVB in the presence of inhibitors for ERK, JNK and p38 kinases and collected cells at 8 h post-treatment for measurement of p53 levels. As shown in Figure 2b, the addition of a specific inhibitor (SB203580) for a p38 kinase substantially reduced p53 induction by UVB. The induction of p53 in the presence of a p38 inhibitor only exhibited twofold compared with eightfold induction in ER-1 cells without an inhibitor. However, addition of either JNK kinase inhibitor (SP600125) or an ERK kinase inhibitor (PD98059) did not affect p53 induction, suggesting that activation of p38 might be required for p53 stabilization after UVB.

To further investigate the role of Gadd45a in the stabilization of p53 protein after genotoxic stress, we employed a human HCT116 Gadd45a-inducible cell line, where Gadd45a expression is controlled by the tet-off system (Jin *et al.*, 2002), and examined p53 induction in the presence of inducible expression of Gadd45a protein. In Figure 3a, HCT116 Gadd45a-inducible cells

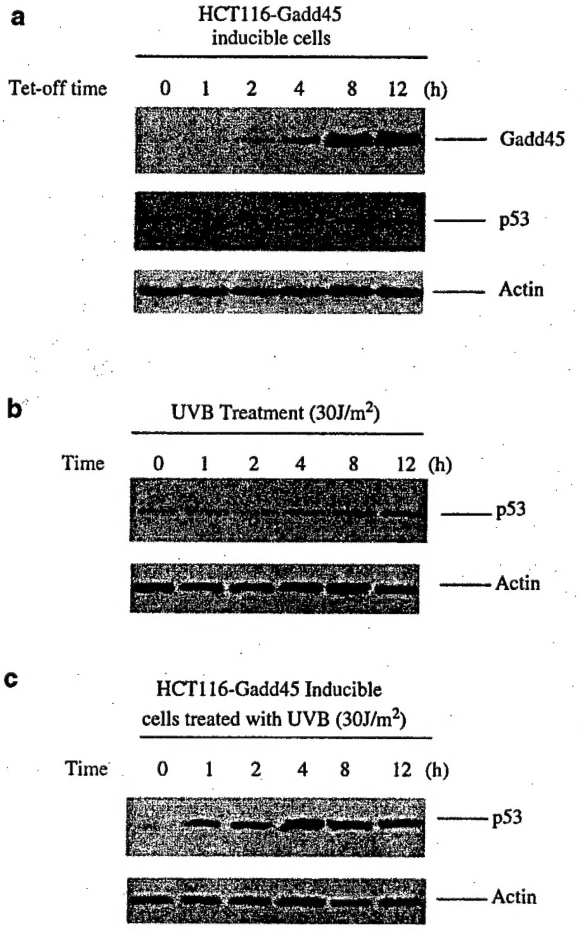

Figure 3 Expression of Gadd45a contributes to stabilization of p53 protein in response to UVB radiation. (**a**) Cellular proteins were prepared from HCT116 Gadd45a-inducible cells after the withdrawal of tetracycline at the indicated time points. Proteins (100 g) were loaded onto SDS–PAGE gel for detection of Gadd45a, p53 and actin. (**b**) HCT116 Gadd45a-inducible cells were grown in the medium containing tetracycline at 2 g/ml, and treated with a low dose of UVB (30 J/m²). Cells were collected at the indicated times for preparation of cellular protein. Proteins (100 g) were loaded onto SDS–PAGE gel for detection of Gadd45a, p53 and actin. (**c**) HCT116 Gadd45a-inducible cells were grown in medium containing tetracycline at 2 g/ml. At 6 h after the withdrawal of tetracycline, cells were exposed to UVB at a dose of 30 J/m² and then collected at the indicated time points for detection of cellular p53 protein

exhibited an extremely low basal level of endogenous Gadd45a protein. After removal of tetracycline, Gadd45a protein was greatly induced. However, inducible expression of Gadd45a alone did not induce p53 protein as p53 remained at the same levels even in the presence of a 10-fold induction of Gadd45a, suggesting that Gadd45a itself is unable to cause the accumulation of p53 protein. We speculated that Gadd45a-mediated p53 induction may also require exposure of cells to genotoxic stress. To demonstrate this, we first induced Gadd45a protein in HCT116 Gadd45a-inducible cells by removing tetracycline. After 6 h, we treated those cells with UVB at 30 J/m^2, a low dose which did not induce p53 (Figure 3b). Cell cultures were collected at the indicated time points and analysed to detect p53 induction. Interestingly, UVB at a dose of 30 J/m^2 was shown to result in p53 accumulation in the presence of inducible expression of Gadd45a. These results indicate that Gadd45a sensitizes cells to a low dose of UVB in terms of p53 induction. Taken all together, the results in the current study demonstrate that Gadd45a plays a role in p53 stabilization after DNA damage.

As discussed earlier, Gadd45a is identified as one of the p53-regulated stress-inducible genes. IR induction of Gadd45a has been shown to depend on normal cellular p53 function, but Gadd45a induction by UV and other non-IR stresses does not require p53. Therefore, there are both p53-dependent and -independent pathways involved in the regulation of Gadd45a induction. Interestingly, the findings in the current study indicate that under some circumstances (cells treated with UVB), Gadd45a may be able to function as an upstream effector that is necessary for the stabilization of p53 protein since p53 induction is diminished in MEF cells with disruption of endogenous Gadd45a. Indeed, Heldesheim *et al.* recently reported that transcriptional activity of p53 following UVB treatment is greatly abrogated in MEFs derived from Gadd45a knockouts.

As to the molecular and biochemical mechanism(s) by which Gadd45a contributes to p53 stabilization, we initially examined whether Gadd45a interacts with MDM2, which associates with p53 and promotes degradation of p53 protein, and releases MDM2 inhibition of p53. In multiple experiments, we found no evidence of an interaction between MDM2 and Gadd45a (results not shown), suggesting that involvement of Gadd45a in p53 stabilization is not through direct interaction of Gadd45a with MDM2. The results in Figure 2a demonstrated that the disruption of Gadd45a affected the phosphorylation of p53 at Ser-15, which is critical for the stabilization of p53 protein. In addition, the findings in Figure 2b showed that p38 kinase, but not JNK and ERK, was involved in the stabilization of p53 protein since the employment of specific inhibitor for p38 kinase substantially abrogated p53 induction by UVB. These observations are supported by other's previous demonstration that p38 kinase is required for phosphorylation of p53 at Ser-15 in response to UV treatment (Bulavin *et al.*, 1999). Interestingly, p38 has not been reported to directly phosphorylate p53 at Ser-15 site. The effect of p38 kinase on Ser-15 is probably medicated through the cooperativity of Ser-33 and Ser-46, at which p38 is able to directly phosphorylate, because Ser-15 phosphorylation by p38 is abolished in the Ser-33/46 double mutant. Most recently, Bulavin *et al.* (2003) has found that Gadd45a protein can physically associate with p38 kinase and is necessary for p38 activation in the presence of Ha-ras . Therefore, the activation of p38 kinase by Gadd45a might be one of the mechanism(s) that are involved in Gadd45a contribution to p53 stabilization. In summary, the current study demonstrates a novel finding that Gadd45a, conventionally as one of the p53-downstream genes, is able to generate a positive 'feedback signal' that is essential for maintaining p53 protein stability and activity in response to genotoxic stress. This positive feedback loop is likely mediated through activation of p38 kinase by Gadd45a (Bulavin *et al.*, 1999).

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